Title: Toxicity in aquatic model species exposed to a temporal series of three different flowback and produced water samples collected from a horizontal hydraulically fractured well.

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Abstract

In the present study, we compared the toxicity and associated chemical characterizations of flowback and produced water (FPW) collected from a single horizontal hydraulically fractured well at different time points during FPW production. Since few studies on whole mixture toxicity related to FPW exist, our aims were to determine both overall toxicity of the FPW mixture in a suite of organisms (*Daphnia magna*, *Lumbriculus variegatus*, *Danio rerio*, and *Oncorhynchus mykiss*) and also determine if toxicity changes depending on variation in FPW chemical properties as a function of time sampled (1.33, 72, and 228 hr FPW samples collected immediately post-well production onset were analyzed in current

study). FPW chemical composition was determined via quadra-pole inductively coupled plasma – mass spectrometry/mass spectrometry (ICP-MS/MS), full-scan high performance liquid chromatography/Orbitrap mass spectrometry (HPLC/Orbitrap-MS), and gas chromatography-mass spectrometry (GC-MS). We observed that FPW sampled later in the production process contained higher ion and total dissolved solids concentrations, whereas the highest concentrations of dissolved organic compounds were observed in the earliest FPW sample analyzed. Toxicity associated with FPW exposure was deemed to be species-specific to a certain extent, but general trends revealed the earliest FPW sampled contained highest toxic potential. Accordingly, we theorize that although the saline conditions of FPW are the foremost toxicological drivers to freshwater organisms, dissolved organics associated with FPW significantly contribute to the overall toxicity of exposed organisms.

Keywords: Aquatic Toxicology; Model Species; Hydraulic Fracturing; Organics; Lethality

1. Introduction

Technological advancements and development in the oil and gas sectors have greatly increased our ability to access world-wide geological reserves of oil and gas previously thought to be unattainable. Implementation of unconventional oil and gas (UOG) recovery processes, in the form of horizontal hydraulic fracturing, is largely responsible for increased production of these resources, and is only expected to become more commonly utilized by industry (Gagnon et al., 2016; Vengosh et al., 2014). UOG operations have significantly expanded in the last decade due to advances in horizontal drilling and completions technology. However, research on environmental impacts of accidental releases is still limited and many gaps in knowledge exist related to the environmental hazards associated to horizontal hydraulic fracturing activities. One such gap pertains to flowback and produced water (FPW), the wastewater by-product that returns to the surface following hydraulic fracturing activities. Although terminology of "flowback" versus "produced water" is subject to operator discretion, FPW collectively is a highly complex mixture composed of original fracturing fluids, compounds from the geological formation itself, saline constituents derived from deep, groundwater reservoirs, and potential daughter

compounds from the transformation of initial fracturing fluid or formation constituents in the high pressure -elevated heat environments of the well-bore-formation interface (Lester et al., 2015; DiGiulio and Jackson, 2016; He et al., 2017a; Hoelzer et al., 2016).

One of the most apparent and immediate hazards posed to the environment from UOG activities and FPW production are surface water contamination events following spills/releases of FPW. In the U.S. alone, 210 billion US gallons (7.95×10^8 m^3) of FPW was produced from 2005 – 2014 (Kondash and Vengosh, 2015). This is especially concerning as a recent study of the Marcellus and Fayetteville shale formations of the eastern U.S. found that on average, wells drilled for hydraulic fracturing purposes were within ~ 300 m of a freshwater surface body of water, such as streams or rivers (Entrekin et al., 2011). Although not as developed as its American counterparts, Canadian UOG development has also been increasing, resulting in larger volumes of FPW produced and increased frequencies of accidental releases to the environment (Alessi et al., 2017; Johnson and Johnson 2012). Thus, understanding effects of FPW releases on the aquatic environment has become essential for government and industry mandates such as spill assessment, clean-up, and remediation protocols. As a result of the paucity of FPW toxicological-related research and high variation in spill mitigation requirements among states and provinces, releases of FPW to surface bodies of water have not historically been considered one of the greatest drivers of FPW environmental risk (Kulander, 2013; Richardson et al., 2013). Furthermore, both Canada and the U.S. do not consider FPW as hazardous waste (Goss et al., 2015). Combining this lack of classification with the decentralized and regionally governed management nature of FPW (Ralston and Kalmbach 2018), policies and mandates dictating FPW spill and remediation strategies are often variable and may not adequately address the hazards posed to the environment. Thus, more conclusive and thorough FPW guideline development among government and industry is a pressing concern. However, to combat the increased risk of FPW environmental impacts, industry operations more commonly implement greater mitigative efforts to assess and reduce risk at the multiple stages of well operations (e.g. pre-development, operational, and well shut-down) by utilization of environmental health and safety regulatory matrices,

more structured and transparent spill reporting and clean-up measures, and more thorough reclamation practices. In Alberta, Canada, this has resulted in a distinct reduction over time in number of reported spills per well (AER Compliance Dashboard) and we assume similar reductions have occurred in other jurisdictions.

Recently, efforts to understand the impact of FPW on aquatic organisms have found that the primary sub-lethal effects observed in organisms following FPW exposure are oxidative and biotransformative stress, negative reproductive impacts, and deleterious effects on the cardio-respiratory system (He et al., 2017b; Blewett et al., 2017a; Blewett et al., 2017b; Folkerts et al., 2017a; Folkerts et al., 2017b). There is also evidence that endocrine disruptive effects may additionally be induced in organisms exposed to FPW (He et al., 2018a; He et al., 2018b). However, references providing strict lethality (measured via varying lethal concentration analyses) toxicological information of FPW on freshwater organisms, as required for guideline development, are still largely unavailable.

One of the complications involved with performing any toxicological study related to FPW is the highly complex and chemically diverse nature of the wastewater (Flynn et al., 2019). Compounding this complexity, the chemical makeup of FPW is highly variable depending on the formation being exploited, initial fracturing fluids used, and well operation characteristics (*e.g.* temperature, pressure, shut-in times, period of flowback) (Kim et al., 2016). Thus, an appreciable amount of work is required to obtain any toxicological knowledge of this wastewater, or of the hazards it poses to the environment. To begin addressing if there are significant toxicological concerns in a legislative and policy-driven context, an evaluation of FPW using standard based whole effluent toxicity protocols (termed WET tests; Chapman, 2000) will provide information about whole-mixture effects on the environment and results subsequently may be included in government and industry protocols and policies.

In the present study, acute FPW toxicity was measured in four different common toxicological model species using samples of FPW collected at different time points during production from a horizontally fractured well in the Duvernay shale play in central Alberta, Canada. These different time

points of sampling cover three operational shifts during the fracturing resource recovery process and FPW produced from these operational phases are hypothesized to contain differing chemical profiles and toxicological potentials, with an increased saline and ion presence predicted in later FPW samples. Early FPW from a producing well is theorized to contain a greater number of initial fracturing additives and may be more toxic to aquatic organisms following exposure. However, FPW samples extending into the production phase of a well (> 120 hrs post well production, as observed in previous studies and those preliminarily made by our group) (Zolfaghari Sharak et al., 2014; Goss et al., 2015) tend to increase drastically in saline content. Among the freshwater aquatic organisms tested, less saline tolerant species (e.g. *Daphnia* and zebrafish) are hypothesized to have greatest sensitivity to FPW, although inter-species responses to FPW exposure are expected to be greatly varied considering the split between invertebrate and vertebrate species and varying lifestyles amongst species tested. Our analyses indicate that temporal variation in FPW chemical characteristics - inorganics, non-targeted organics, and polycyclic aromatic hydrocarbons (PAHs) - can significantly alter toxicity to organisms, and that there is great inter-species variability in lethality responses to FPW. Our chemical characterization and toxicological results further the notion of FPW complexity and serve as a reminder that species-specific differences should be considered for understanding the implications of accidental FPW releases to the environment.

2. Materials and Methods

2.1. FPW Samples and Animal Maintenance

All FPW samples used for analyses were provided by Encana Services Company Ltd. (Calgary, AB, CAN). FPW samples were collected from a single horizontal hydraulically fractured well post-stimulation. Three timepoints for FPW collection (and subsequent analysis) were chosen: a 1 hour, 20 min sample (1.33 hr), a 3-day sample (72 hr), and a 9-day, 12 hour sample (228 hr) (see Table 1). A saline solution matching the major cation and anion concentrations of the most saline FPW sample (228 hr) was also created to control for saline-induced responses (see SI Table 1 for ionic composition information).

A total of four model test species were chosen for toxicological experimentation, including two invertebrate and two vertebrate species. Our two chosen invertebrate species included the freshwater cladoceran, *Daphnia magna* (neonates), and the oligochaete *Lumbriculus variegatus*. Our two vertebrate species were zebrafish (*Danio rerio*; embryos) and rainbow trout (*Oncorhynchus mykiss*; embryos and juveniles). All invertebrate species were cultured and maintained in dechlorinated City of Edmonton (Edmonton, AB, CAN) tap water (moderately hard: $[Na^+]$ = 14.6 mg/L, $[Ca^{2+}]$ = 55.9 mg/L, $[Mg^{2+}]$ = 15.3 mg/L, $[K^+]$ = 2.5 mg/L, pH: 7.9, conductivity: 395 ± 0.5 µS/cm, dissolved oxygen: 7.5 ± 0.5 mg/L, general hardness: 186 mg/L as $CaCO_3$, salinity: 0 ppt) at 20 ± 1 °C. Zebrafish embryos (wildtype strain AB) were collected from adult fish housed in 30 L tanks (~ 25 fish/tank, 14 hr light: 10 hr dark photoperiod) in the University of Alberta zebrafish aquaculture facility filled with zebrafish facility water (pH: 7.4, conductivity: 553 µS/cm, temperature: 28.5 ± 1 °C, dissolved oxygen: 7.3 mg/L, hardness: 174 mg/L as CaCO3, salinity: 0 ppt) treated by a RiOs 100 (reverse osmosis) water purification system. Diploid rainbow trout embryos were collected from the Raven Creek Trout Brood Station (Caroline, AB, CAN), brought to the University of Alberta, and maintained at 10 °C in heath-tray stacks connected to a recirculating water chiller apparatus containing 150 L of dechlorinated City of Edmonton tap water filtered and treated by a bio-ball purification layer (to treat for nitrogenous waste products) and ultra-violet light sterilization. Recirculating water for rainbow trout embryos was constantly aerated and maintained at dissolved oxygen levels of 8.1 ± 0.3 mg/L. Periodic water top-ups to the heath-tray-recirculation system were additionally performed. Juvenile rainbow trout (2.38 ± 0.15 g) were maintained in aerated flow-through 400 L tanks filled with dechlorinated City of Edmonton tap water at 10 ± 1 °C. All animal use was approved by the University of Alberta Animal Care Committee under protocols AUP00001334 and AUP00002352. All previously stated variation was calculated to one standard deviation.

2.2. Inorganic FPW Characterization

2.2.1. Inductively Coupled Plasma (ICP) -MS/MS Analysis

ICP-MS/MS analysis was used to quantify the concentration of cations, bromide, and total sulfur (Figure 1, SI Table 1). Before analysis all samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 850 for Na analysis and 85 for all other elements with 18 MΩ·cm ultrapure water. Diluted 10 ml samples were then acidified with 12 µL using 15.698 N trace metal grade nitric acid. For analysis, an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) was used with a RF power of 1550 W, a RF reflected power of 18 W, a microMist nebulizer and nickel/copper cones. Samples and external standards were analysed in high matrix mode, in which samples were diluted inline with 8 mL/min argon. Additionally, analysis was performed in MS/MS mode for greater mass resolution and a collision gas reaction cell was used with He gas (3 mL/min), O_2 gas (10% max flow) or H_2 (5 mL/min) gas to overcome matrix interferences (SI Table 2.). Instrumental drift was accounted for using 0.5 ppm solution of indium, which was added to each sample using an inline internal standard addition system. For quality assurance and control, a standard solution was run at the start, middle, and end of each run (every 10 sample for n = 3) to quantify the run precision and percent recovery for each element (SI Table 2).

2.2.2. Anion, total organic carbon and total nitrogen analysis

Ion chromatography was used to determine the concentration of chloride (Figure 1, SI Table 1). Before analysis, samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 2000 using 18 MΩ·cm ultrapure water. Analysis was then performed on a Dionex Ion chromatography DX 600 with a 4mm analytical column (AS9-HC), guard column (AG9-HC), and a 4 mm ASRS Ultra suppressor. For total non-purgeable organic carbon (TOC) and total nitrogen (TN) a subsample of the filter sample was diluted by a factor of five and analyzed using on a TOC analyser (Shimadzu model TOC-V-CHS/CSN). For quality assurance and control for IC a control standard solution was run every five samples (n=4) to quantify the run precision and percent recovery for each element (SI Table 3).

2.3 Organic FPW Characterization

2.3.1. Extraction of Organics

For each FPW sample, 150 mL of FPW was vacuum filtered through a glass fiber membrane (90 mm diameter, pore size: 0.4 μm), then subsequently freeze-dried for 48 hrs. Approximately 50 mL of the aqueous filtrate was then liquid-liquid extracted using a repeated process of 20 mL of dichloromethane (DCM) and shaking for 3 minutes twice. The combined extracts (40 mL) for each sample were concentrated by nitrogen gas evaporation and reconstituted in 1 mL methanol for High Performance Liquid Chromatography/Orbitrap Mass Spectrophotometry (HPLC/Orbitrap-MS) analysis. The internal standard mix (10 ng of each, Wellington Laboratories, ON, CAN) was spiked into the remaining (unextracted) 100 mL of aqueous filtrate, extracted and evaporated as described above, and reconstituted in 3 mL hexane for further cleanup.

Accelerated solvent extraction (ASE) was used for PAH extraction from particulates in the freeze-dried glass filters sediments. A different, separate glass fiber filter was added at the bottom of the extraction cell, and ~ 2-3 g of florisil (pre-cleaned by DCM) was added, followed by dried sediment filters, to which the internal standard mix was then spiked (10 ng of each). ASE cells were filled with solvent (hexane/DCM 4:1 v:v; Optima™, Fisher Scientific, NH, USA), pressurized to 14 MPa, and heated to 80 °C within 6 min. Pressure and temperature were held for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s. This extraction cycle was repeated once more. Approximately 40 mL of total extract was gently concentrated by nitrogen gas evaporation and reconstituted in 3 mL of hexane for further cleanup.

2.3.2. High Performance Liquid Chromatography/Orbitrap Mass Spectrophotometry (HPLC/Orbitrap - MS)

Dissolved organic compounds in FPW aqueous filtrate extracts (section 2.3.1.) were analyzed via HPLC/Orbitrap-MS. Briefly, 10 μL of diluted organic extract (equivalent to 50 μL of the respective original FPW sample) was used for targeted organic compound identification and semi-quantification using reverse phase LC-Orbitrap-MS. Orbitrap MS was operated in positive electrospray ionization mode, and acquisition was in full scan mode (*m/z* 100 to 2000) at 2.3 Hz, with resolving power set to 120,000 at

197 *m/z* 400. Tandem mass analysis of targeted compounds was also performed using collision-induced
198 dissociation or high collision dissociation. Please see He et al. 2018a for further details. The identification
199 of target compounds, as listed in Figure 2, was achieved through accurate mass measurement and tandem
200 mass spectra and later confirmed by the corresponding reference standards. The semi-quantification was
201 achieved by an external linear calibration curve analysis of each of the reference standards. Please see
202 Sun et al. 2019 for a more in-depth account of orbitrap characterization, reference standard chemical
203 concentrations and recoveries, and all other quality assurance and control information.

204 ***2.3.3. Polycyclic Aromatic Hydrocarbon (PAH) Analysis***

205 In order to characterize and measure FPW sample PAHs, copper powder and anhydrous sodium
206 sulfate (pre-cleaned with DCM) were added into the 3 mL extracts (section 2.3.1.) and vortexed. Solid
207 phase extraction was used for cleanup of all the sample extracts. A Sep-Pak Silica 6 cc Vac cartridge (1 g;
208 Waters, MA, USA) was conditioned with 5 mL solvent (hexane/DCM 7:3 v:v; Optima™, Fisher
209 Scientific, NH, USA), followed by 5 mL of hexane. Extracts (3 mL) were loaded into cartridges and
210 washed with 4 mL of hexane. PAHs were finally eluted with 5 mL of hexane/DCM 7:3 (v/v) which was
211 subsequently concentrated to 200 μL for gas chromatography-mass spectrometry (GC-MS) analysis.
212 Details of PAH standards, internal standard corrected recoveries of the detectable PAHs based on
213 accelerated solvent extraction method (range from 60.0–114%) and liquid-liquid extraction (range from
214 82.9–179%), and the GC–MS instrumental method have been described in previous work (Zhang et al.,
215 2016). Similarly, details of PAH analyte detection limits can be found in He et al. 2018b.

216 ***2.4. Lethal Concentration (LC) Analyses and Toxicity Determination***

217 LC analyses (LC_{10}, LC_{20}, and LC_{50}) were performed according to Organisation for Economic Co-
218 operation and Development (OECD) guidelines (OECD-202, 2004; OECD-203, 1992; OECD-236, 2013)
219 with some slight adjustments. Dechlorinated City of Edmonton tap water was used for all exposures and
220 FPW dilutions. Fluorescent lighting on a ratio of 14:10 hr light:dark was applied for all exposures. For
221 96-hr LC analyses, static-renewal exposures were employed where 50% solution changes were made

every 48 hrs. Dead organisms were immediately removed from exposure containers. A total of seven different FPW concentration dilutions (created by mixing raw FPW with dechlorinated tap water) - including a freshwater control - were used for each LC analysis with six replicates for each individual FPW time-point sample at each dilution being employed to ensure statistical robustness. A series of range-finding tests were performed for all species prior to acute lethal toxicity analyses to ensure proper dilutions of FPW were being used to accurately capture LC values of each FPW sample.

All invertebrate exposures were acute 48-hr, static exposure lethal analyses occurring in 30 mL of FPW containing mediums at 20 ± 0.5 °C in 50 mL glass beakers (10 organisms/beaker). *Daphnia* neonates were immediately collected from adult organisms and exposed within 24 hrs following adult brooding. Adult *Lumbriculus variegatus* were collected from cultures and held overnight before being used in bioassays. Fish (both zebrafish and rainbow trout) embryo lethal analyses occurred over a 96-hr, static-renewal exposure period and used ten embryos/beaker for exposures. Observations every 24 hrs under a stereomicroscope were performed to determine mortality. Zebrafish embryos collected from adult fish were immediately placed into petri dishes containing fresh zebrafish facility water and were subsequently observed under a Leica Zoom 2000 stereomicroscope (Leica Camera Co., GER) to determine embryo fertilization. After confirming fertilization (~ 2 hours post fertilization; hpf) and viable status, embryos were transferred to 100 mL of respective FPW solutions in 250 mL glass beakers maintained at 25 ± 0.5 °C for 96-hr LC_{50} analyses.

Rainbow trout embryos (7 – 12 days post fertilization; dpf) removed from the recirculating heath-tray chiller unit were immediately placed into 200 mL of respective FPW solutions in 400 mL beakers maintained at 10 ± 1 °C under constant aeration for LC analyses. Observations every 24 hrs under a stereomicroscope were performed to determine embryo mortality. For juvenile rainbow trout 96-hr LC_{50} analyses, static-renewal exposures occurred in 10 L glass tanks containing 8 L of respective FPW solutions under constant aeration and were maintained at 10 ± 1 °C. All tanks were allowed to equilibrate with FPW exposure solutions 24 hrs prior to onset of LC analyses. Fish were fasted for 24 hrs prior to experimentation and were not fed for the duration of the 96-hr LC analysis. For each exposure, 7 fish

were used per tank at each concentration. Observations every 24 hrs were performed to determine fish mortality.

2.5. Statistical Analyses and Calculations

All LC analyses and associated 95% confidence intervals (CI) were calculated using a three-parameter probit model (TRAP; Toxicity Relationship Analysis Program, v1.30a, U.S. EPA). Compared LC values which did not have overlapping 95% CIs were considered significantly different. All graphing and non-linear curve-fitting procedures were performed using the statistic and graphing program Prism (GraphPad Software Inc., CA, USA).

3. Results

3.1. Inorganic Characterization of FPW

ICP-MS/MS inorganic analyses revealed a predicted increasing ion concentration gradient with increased FPW sampling period. This was highlighted in many of the major salt ion characterizations; ions such as Cl, Na, Ca, K, and Mg, which were all shown to increase in concentration as the timing of FPW sampling progressed (Figure 1, SI Table 1). Accordingly, a similar increase in total dissolved solid (TDS) readings was observed, as the highest recorded value was found in the 228 hr sample at ~175700 mg/L. Interestingly, total organic carbon (TOC) values dropped as FPW sampling progressed (SI Table 1), while total nitrogen (TN) measurements did not follow any apparent trend. Regarding metals, high concentrations of Fe, Sr, and Ba were found in all FPW samples, although only Sr displayed a rough observable trend of increasing concentration with FPW sampling time (SI Table 1). Outside these constituents, Br was also found in FPW samples at high concentrations (up to ~ 270 mg/L), but with no obvious trend (SI Table 1).

3.2 Organic Characterization of FPW

Following HPLC/Orbitrap-MS/MS analyses, the organic chemical profiles revealed distinct differences between FPW sampling periods. However, the organic composition of FPW is complex and the lack of reference standards makes quantification difficult, thus, only the organic compounds that were

identified with high confidence (i.e. comparison with corresponding reference standards) are semi-quantified and presented in this study. Polyethylene glycols (PEGs) were found in all FPW samples, with the highest concentrations found in the 1.33 hr sample (63.5 ng/L; Figure 2). Similarly, C_{10}-alkyl ethoxylates (C_{10}-AEO) and octylphenol ethoxylates (OPE) were present in all FPW samples, with the highest concentration of C_{10}-AEO (111 ng/L) found in the 1.33 hr sample and the concentration of OPE highest in the 72 hr FPW sample (85.2 ng/L). Beyond these chemicals, triphenyl phosphate, tri (2-butoxyethyl) phosphate, 3-(dodecanoylamino)-N,N-dimethylpropan-1-amine oxide, and 2-[dimethyl-[3-(tetradecanoylamino)propyl]azaniumyl]acetate were found at varying levels across the FPW samples analyzed, although all were found at very low concentrations overall (Figure 2).

HPLC-MS/MS analyses of 22 PAHs (16 identified by the USA EPA as priority PAHs of toxicological concern; Office of the Federal Registration, 1982) in the aqueous and sediment phases of the FPW samples revealed that all samples contained PAHs; the majority being either of 3 and 4-ringed analytes (Table 2). In particular, the 3-ringed PAH phenanthrene was commonly found at the highest concentrations, with largest total (aqueous and sediment phase summation) concentration of 36.9 µg/L phenanthrene found in the 1.33 hr FPW sample. Phenanthrene (and phenanthrene related compounds such as 1- methylphenanthrene and 3,6-dimethylphenanthrene) were also found to compose the majority of the PAH compounds present in FPW samples, comprising 66%, 63%, and 71% of the 22 total PAHs analyzed in the 1.33 hr, 72 hr, and 228 hr FPW samples, respectively (Table 2). Overall, all PAH compounds followed the general trend of having the highest concentration in the 1.33 hr sample, and lowest in the 72 hr FPW sample, resulting in total PAH concentrations of 112.7, 11.6, and 25.1 µg/L in 1.33 hr, 72 hr, and 228 hr FPW samples, respectively.

3.3. Aquatic Species Lethality Responses

For clarity and succinctness, all statements of LC analyses and discussion of results will pertain to LC_{50} analyses unless otherwise stated. However, information on LC_{10} and LC_{20} analyses can be found in Table 3. From our inorganic analyses, we anticipated the inherently high salinity of FPW to be a significant contributor to the toxicological hazard of FPW spills posed to freshwater aquatic organisms.

However, our organic chemical characterizations also revealed a substantial amount and diversity of organic compounds to be present. Thus, when compared to our saline control exposures, not all organismal toxic responses to the experimental FPW series samples are observed to be strictly implicated to the high salt concentrations of the FPW samples.

Daphnia magna responses to our FPW samples were observed to be most sensitive of the four species tested and displayed the greatest acute toxicity regardless of sample collection timepoint. Comparing the daphnid LC_{50} FPW dilution values of 1.58, 0.76, 0.94, and 2.81% for our 1.33 hr, 72 hr, 228 hr, and salt control, respectively (Figure 3, Table 3), to the observed LC_{50} values observed in rainbow trout embryos (8.82, 12.26, 10.83, and 16.87% for 1.33 hr, 72 hr, 228 hr, and salt control, respectively) highlights the large variability in aquatic organism response to FPW. Relatively moderate LC_{50} responses of zebrafish embryos (3.03, 2.22, 2.15, and 2.11%), *Lumbriculus* (3.13, 4.18, 3.80, and 4.11%), and juvenile rainbow trout (7.14, 10.51, 9.02, and 14.24%) when exposed to the FPW series (1.33 hr, 72 hr, 228 hr, and salt control, respectively) (Figure 3, Table 3) were additionally observed. These results again highlight both the diverse mechanisms and toxic nature of FPW solutions.

4. Discussion

A significant contribution to the differences in toxicity between the FPW samples analyzed can be attributed to noted differences in inorganic and organic chemical profiles. The FPW samples chosen for analysis from the horizontal hydraulically-fractured well studied spanned a time series hypothesized to hold varying toxicological potentials, as all three time points represented a shift in general well production states.

When individually assessing toxicity amongst species, it was observed that *Daphnia magna* were overall the most sensitive species (lowest LC values) of those tested to FPW samples at all collection time points (Figure 3, Table 3). Interestingly, *Daphnia* were most sensitive to our 72 hr FPW sample when all other species (save for zebrafish embryos) were least sensitive to this particular sample. However, differential *Daphnia* responses to the three FPW samples were insignificant (overlapping LC_{50} 95% confidence intervals), but all elicited significantly higher toxic responses (lower LC_{50} values with non-

overlapping 95% CIs) compared to the salt controls, indicating that something besides salinity in FPW (presumably organics) causes an additional significant toxic effect. Zebrafish embryos were found to be the second most sensitive to FPW exposures amongst all species tested. However, zebrafish embryo responses generally contrasted other species responses, as the saline control elicited the greatest toxicity in zebrafish embryos and was significantly more toxic compared to the 1.33 hr sample (non-overlapping LC_{50} 95% CIs). These results suggest that zebrafish embryos are, above all, most sensitive to high osmotic conditions, and that salinity dominated to a greater degree FPW toxicity in zebrafish embryos, perhaps masking any other effects. Considering their current natural ecological niche and no recent evidence of a diadromous lifestyle within the evolution of their species (Engeszer et al., 2007; Imoto et al., 2012), the high sensitivity of zebrafish to hyperosmotic conditions is a logical response. *Lumbriculus* were found to be the third most overall sensitive species to FPW exposure, although general trends of sensitivity in this species were mirrored in both embryonic and juvenile rainbow trout exposures (Figure 3, Table 3). When analyzing these organism response trends to FPW exposure, it was observed that the 1.33 hr sample elicited greatest toxicity, while the 72 hr sample was least toxic. In contrast to zebrafish, the family Salmonidae, of which rainbow trout are a member, have a relatively recent evolutionary history of anadromy (natural transfer between freshwater and seawater environments) (Docker and Heath, 2003; Crespi and Fulton, 2004; Alexandrou et al., 2013). Thus, a more saline-tolerant response to FPW was expected and found for rainbow trout. Interestingly, responses to different FPW samples within each distinct rainbow trout exposure series were significantly different from one another (no 95% C.I. overlap for LC_{50} analyses). However, juvenile rainbow trout were observed to be significantly more sensitive to FPW exposures compared to embryonic forms; a result consistent with earlier reports suggesting that embryonic life-stages are often more resistant to environmental stressors than larval stages (immediately following hatching) and juvenile forms (Embry et al., 2010; Woltering, 1984). It is thought that the enveloping chorion *in ovo* acts as a barrier to toxicants and other environmental stressors, thereby protecting the developing organism within the embryo (Embry et al., 2010; Cotelli et al., 1988; Pelka et al., 2017; Denluck et al., 2018).

As demonstrated by the total ion chromatograms of HPLC/Orbitrap-MS and PAH analyses of FPW extracts, there are clear differences in both diversity and abundance of organic compounds among samples (Figure 2, Table 2). Of the semi-quantified compounds identified, class C_{10}-AEO non-ionic surfactants were highest in our 1.33 hr sample. This particular class of surfactants have known bioaccumulation potentials (Müller et al., 1999; Cheng et al., 2005), with nonspecific narcosis as the suspected primary toxicological mode of action (Dorn et al., 1997; Müller and Escher, 1999). Another semi-quantified class of surfactants identified were OPEs. These are non-ionic surfactants which may mimic endogenous hormones and cause endocrine disruption upon exposure (White et al., 1994; Nimrod and Benson, 1996). These surfactants are degraded/or metabolized into octylphenols (OPs), which are often more toxic and more persistent in the environment (Ying et al., 2002; Ahel and Giger, 1993). Although the concentrations of C_{10}-AEOs were below water quality guidelines of the Canadian Environmental Protection Act (70 µg/L; Environment and Climate Change Canada Environmental Protection Act, 1999) and OPEs in the 1.33 hr sample were lowest amongst the FPW samples, the toxicity of surfactants in the 1.33 hr sample may still play a larger role in producing greater effects compared to the other two FPW samples, particularly when other characteristics of the mixture are taken into account. Specifically, PEGs were found at highest levels in the 1.33 hr sample. Although not inherently toxic to fish individually, PEG physiochemical properties may alter the toxicity of other compounds found in the FPW mixture. In relation to surfactants, PEGs are shown to increase surfactant critical micelle concentrations (CMC), decrease average micelle aggregation (N_{agg}), and increase surfactant polydispersity (Nagarajan and Wang, 2000), while surfactants which incorporate PEGs directly into their chemical structure (such as tocopheryl polyethylene glycol succinate surfactants; TPGS) increase CMCs and decreased N_{agg} values compared to traditional non-ionic surfactants without PEGs associated (Sadoqi et al., 2009). These TPGS surfactants have been shown to be an effective pharmaceutical agent for drug deliveries by increasing drug absorption in tissues (Zhang et al., 2012; Ismailos et al., 1994). Increased PEG presence in our 1.33 hr FPW sample may therefore increase the bioavailability of the surfactants

present in the 1.33 hr sample and allow them to interfere and react to a greater degree with biological surfaces of exposed organisms to induce greater toxicity.

PAH analysis of the current samples similarly affirmed TOC analyses depicting the 1.33 sample to contain the greatest amount of organic compounds (Table 2). Of the PAH compounds investigated, all present/detectable forms were highest in concentration in the 1.33 sample, resulting in the highest Σ16 and Σ22 PAHs concentrations. Specifically, phenanthrene-related PAH compounds were highest in the 1.33 hr sample, although in both the 72 and 228 hr samples, these were also the major PAHs identified. PAHs are known to cause numerous toxic effects in exposed organisms. Although PAH toxicity can be species dependent, given that invertebrates are generally considered to exhibit lower PAH metabolic and elimination capabilities, other factors such as developmental stage are also known to influence PAH toxicity (Meador et al., 1995; James, 1989; Varanasi et al., 1989). Regardless, toxicity associated with PAH exposure may come from the parent molecule itself or, as is more often the case, from metabolite intermediates formed during biotransformation processes. These intermediates may include highly reactive epoxides formed during phase I cytochrome P450 (CYP) monooxygenase oxidation steps which may also be transformed into toxic phenol derivatives depending on the original substrate (Buhler and Williams, 1988; Livingstone, 1998). The creation of these intermediate metabolites through CYP systems (and the effects they have on biological systems) are mediated at differing levels depending on the specific PAH by as many as 70 nuclear receptors, including the aryl hydrocarbon receptor (AhR), retinoid X receptor (RXR), and others (Honkakoski and Negishi, 2000; Xu et al., 2005).

Alternatively, PAHs may non-specifically interfere with other cellular processes via nonspecific narcosis (van Wezel and Opperhuizen, 1995; Barron et al., 2004; Di Toro et al., 2007) or other cellular processes/receptors not yet studied. PAHs are also known to produce oxidative stress responses in both invertebrates and vertebrate species (Dalton et al., 2002; Sun et al., 2006; Lemaire et al., 1994; Penning et al., 1996; Zangar et al., 2004). Considering phenanthrene, phenanthrene-related compounds, and other 3-ring PAHs (such as fluorene and dibenzothiophene) in our FPW samples are generally found at the highest concentrations compared to other PAHs, we suspect that significant cardiotoxic potentials are

403 associated with FPW, as recent research often associates ‘blue-sacs disease’ (a condition characterized by
404 larval craniofacial and tail/spine deformities, as well as pericardial and yolk-sac edemas) (He et al., 2012;
405 Spitsbergen et al., 1991) with exposure to these 3-ring PAHs in larval fish species. Exposure to 3-ring
406 PAHs in fish has recently also been shown to directly alter cardiomyocyte function by specifically
407 affecting Ca^{2+} and K^{+} currents via blockade of K^{+} channel and disruption of sarcolemmal and sarcoplasm
408 reticulum Ca^{2+} cycling (Brette et al., 2017; Brette et al., 2014). Detriments to cardiac function would
409 undoubtedly place higher energetic demands and stress in exposed organisms and may be another
410 toxicological hazard warranting assessment when releases of FPW occur.
411
412 ***5. Conclusion***
413 Research on FPW and wastewaters associated with hydraulic fracturing activities has only
414 recently begun in a manner which assesses the toxicological hazards associated with such fluids. In this
415 study, we aimed to not only determine baseline toxicities of FPW in multiple relevant toxicological model
416 species, but also determine how toxicity (and chemical characterizations) change depending on duration
417 of early stage flowback from a well. These data are of value to the reassessment of risk and remediation
418 strategies/requirements and will overall contribute to a better understanding of the potential impacts of
419 FPW releases. Despite the complexity and variations in FPW chemical and toxicological makeup
420 (dependent on numerous geological and operational factors), understanding basic toxicological and
421 chemical characteristic tendencies will help shape governmental and industrial FPW management policies
422 and hazard assessments.
423 In the present study, we have determined that FPW toxicity is not only species-dependent but is
424 also influenced by the length of time that a well has been producing FPW. A significant component of the
425 toxicity of FPW to freshwater organisms is mediated by the high salinity and organic make-up (from
426 either initial fracturing fluid constituents and formation derived organics) of the wastewater, with even
427 very high dilutions of FPW inducing toxicity in all species tested. However, all species also demonstrated
428 significantly different toxic responses to at least one of the FPW samples tested separate of saline-related

effects, suggesting that other components of FPW are contributing to overall toxic potentials. In particular, earlier samples of FPW collected closer to the beginning of the flowback period (*e.g.* our 1.33 hr sample) were determined to contain the highest organic contaminant concentrations and were also generally most toxic to the organisms studied. Considering these earlier samples contained overall lower salt concentrations, and metal concentrations that were either lower or equal to later FPW samples, it is assumed that organic contaminants were responsible for most of the added toxicity observed. However, all FPW samples contained significant toxic potential and should be considered hazardous regardless of time post-well stimulation when accidental releases to the environment occur.

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